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(54) Title: USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

(57) Abstract

Hematopoietic precursor cell numbers are increased by exposing the cells to leptin. The hematopoietic precursor cells, such as stem cells or progenitor cells of a lin-/c-kit+ fraction, can be separated from mature hematopoietic cells present in a blood product, such as bone marrow, prior to exposure to leptin. The number of hematopoietic stem cells *in vitro* can also be maintained by exposure to leptin, and primitive hematopoietic stem cells can be sustained *in vitro* without substantial proliferation or differentiation. Also, the IL-3 mediated stimulation of hematopoietic cells can be inhibited by treatment with leptin. A composition containing leptin is used to sustain or expand the number of hematopoietic precursor cells. These methods and compositions can be used to support the survival of stem cells during collection and processing, to mobilize stem cells to peripheral circulation, to expand progenitors *in vitro*, to improve bone marrow transplantation, and to increase the efficiency of gene therapy which is targeted to long term repopulating hematopoietic stem cell (LTR-HSC) populations.

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USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

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Background of the Invention

I. Leptin, the ob gene product

The recently cloned mouse obesity (ob) gene encodes
10   an adipose tissue-derived signalling factor for body weight
homeostasis. Zhang et al., Nature 372:425-432 (1994). The ob
gene product was subsequently named "leptin," which means thin
in Greek. The leptin protein is proposed to function as part
of a signalling pathway from adipose tissue that regulates the
15   size of body fat deposits. ob/ob mutant mice are hyperphagic
and maintain a body mass eight times of the wild type animal.
The leptin molecule is 167 amino acids long and
appears to be well conserved among vertebrates. A cDNA clone
encoding human leptin has been isolated and the amino acid
20   sequence shows a high degree of identity between human and
mouse. mRNA for leptin is only expressed in white adipose
tissue (WAT), and leptin has the properties of a secreted
hormone. The fact that leptin is secreted only from fat
tissue, taken together with the results from biological
25   studies of the ob/ob mice, strongly supports leptin's role as
a satiety factor. Administration of recombinant leptin
reduces food intake and increases energy expenditure in ob/ob
mice (Halaas et al., Science 269: 543-546 (1995); Campfield et
al., Science 269: 546-549 (1995); Pelleymounter et al.,
30   Science 269: 540-543 (1995)), suggesting that it signals to
the brain the magnitude of fat stores.
The receptor for leptin has been identified by an
expression cloning method (Tartaglia et al., Cell 83: 1263-
1271 (1995)). It is a single transmembrane-spanning receptor
35   that appears most related to the gp130 signal-transducing
component of the IL-6 receptor, a signal transducer shared by
the IL-6, LIF, OM, and IL-11 receptor systems. Strong

evidence indicate that the diabetes (db) gene encodes the
leptin receptor (Chen et al., Cell 84: 491-495 (1996); Chua et
al., Science 271: 994-996 (1996); Lee et al., Nature 379:632-
635 (1996)) and that the db/db mutation is the result of
5   abnormal splicing of the leptin receptor. At least five
alternatively spliced mRNA have been identified in mouse and
four in humans, with only one form (the long form) having a
signal transducing cytoplasmic domain (Lee et al., supra).

        The leptin receptor has a much broader tissue
10   distribution than leptin, and has been detected most strongly
in lung and kidney, (Tartaglia et al., supra). With a much
more sensitive method (RT-PCR), mRNA for leptin receptor can
also be detected in brain, hypothalamus, testis and adipose
tissue (Lee et al., supra). A "B219" receptor isolated from
15   yolk sac cell lines, the expression of which was also observed
in bone marrow and fractionated bone marrow, has been
identified as the leptin receptor. Cioffi et al., Nature Med.
2: 285-589 (1996). Ghilardi et al., Proc. Natl. Acad. Sci.
USA 93:6231-6235 (1996), has reported the presence of leptin
20   receptor sequence in bone marrow and many other lymphoid
tissues. The receptor appears to be abundant in peripheral
and mesenteric lymph nodes.

        In several rodent models of obesity, leptin mRNA
expression and the level of circulating leptin are increased
25   (Frederich et al., Nature Med. 1: 1311-1314 (1995); Maffei et
al., Proc. Natl. Acad. Sci. USA 92: 6957-6960 (1995); and
Ogawa et al., J. Clin. Invest. 96: 1647-1652 (1995)). One
study reports that a high-fat diet evokes a sustained increase
in circulating leptin in both normal and transgenic mice, with
30   leptin levels accurately reflecting the amount of body lipid
across a broad range of body fat (Frederich et al., supra).
However, despite increased leptin levels, animals fed a
high-fat diet became obese without decreasing their caloric
intake, suggesting that a high content of dietary fat changes
35   the "set point" for body weight, at least in part by limiting
the action of leptin.

Studies have suggested that obese humans and
rodents (other than *ob/ob* mice) are not defective in their
ability to produce leptin mRNA or protein and generally
produce higher levels than lean individuals (Considine et al.,
5   J. Clin. Invest. 95: 2986-2988 (1995); Lonnqvist et al.,
Nature Med. 1: 950-953 (1995); Hamilton et al., Nature Med. 1:
953-956 (1995), Considine et al., N. Engl. J. Med. 334: 292-
295 (1996); and Rohner-Jeanrenaud and Jeanrenaud, N. Engl. J.
Med. 334: 324-325 (1996)). These data suggest that resistance
10 to normal or elevated levels of leptin may be more important
than inadequate leptin production in human obesity.

II. Hematopoietic stem cells (HSC).

The hematopoietic system contains a core of stem
15 cells (HSC) that can expand dramatically on demand and can
differentiate to provide all of the myelolymphoid lineages of
the circulating population. Till and McCullough, Rad. Res.14:
213-223 (1961), identified a transplantable cell that could
produce millions of descendants (including cells with
20 clone-forming capacity) upon lodgment in the spleens of
lethally irradiated mice (Siminovitch et al., J. Cell Comp.
Physiol. 64: 23-36 (1964)). This transplantable colony
forming cell (CFU-S) is now known to consist of subpopulations
with defined capacities to replicate and form specific
25 lineages (Magli et al., Nature 295: 527-529 (1982); Wolf and
Priestley, Exp. Hematol. 14: 676-689 (1986)).

In recent years, it has been clear that there must
exist a still more "primitive" HSC that does not manifest
itself as a CFU-S (the "pre-CFU-S") (Hodgson and Bradley,
30 Nature 281: 381-382 (1979); Pietrzyk et al., Mech. Ageing Dev.
49: 79-86 (1989); Ploechmacher and Brons, Exp. Hematol. 17:
263-266 (1989)). The more primitive this HSC is, the more
restricted is its ability to divide under steady-state
conditions. A number of attempts have been made to purify
35 these cells using various methods, which have typically
included a FACS selection step based either upon cell surface
antigenic or lectin determinants or upon differential

affinities for mitochondrial- or DNA-binding vital dyes
(Bartelmez et al., Exp. Hematol. 19: 861-862 (1991)).

   The cells fractionated by these methods have been
studied for proliferative capacity, pluripotency and purity
5   using such measurements as capacity to proliferate and
differentiate *in vitro* and/or *in vivo*. The most stringent of
these tests is the ability to repopulate all myelolymphoid
lineages of a lethally irradiated animal (Metcalf and Moore,
Haematopoietic Cells, Frontiers of Biology Series, North
10   Holland, Amsterdam (1971), p. 71. Other approaches have used
the transplantation of unfractionated but genetically distinct
marrow donor cells, based on either alloenzymes or the random
insertion sites of retrovirally transferred genes (Harrison et
al., Proc. Natl. Acad. Sci. USA 85: 822-827 (1988); Abkovitz
15   et al., Proc. Natl. Acad. Sci. USA 87:9062-9067 (1990); Dick
et al., Cell 42: 7179 (1985); Keller and Snodgrass, J. Exp.
Med. 171: 1407-1419 (1990); Jordon and Lemischka, Genes Dev.
4: 220-228 (1990)). These studies, as well as studies which
used elutriation methods for marrow cell fractionation (Jones
20   et al., Nature 347: 188-190 (1990)), demonstrated the
heterogenous nature of the stem cell compartment with respect
to the length of time over which individual clones may
function, and the lineage potential of these clones. Distinct
HSC subpopulations were demonstrated that ranged from the
25   ability to short-term (weeks to months) repopulate (STR-HSC)
to longterm (> 1 year) repopulate (LTR-HSC) lethally
irradiated mice (see Bartelmez et al., supra).

   Methods have been developed to isolate and separate
the subpopulations of LTR-HSC and STR-HSC to an extremely high
30   level of purity (as judged by both *in vivo* and *in vitro*
assays). The ability to repopulate transplanted mice and to
proliferate clonally *in vitro* have been studied (Wolf et al.,
Exp. Hematol. 21: 614-622 (1993)). One LTR-HSC is capable of
replenishing the marrow of a lethally irradiated mouse. This
35   is accomplished by administering a single LTR-HSC from a male
mouse along with STR-HSC from a female mouse to a lethally
compromised female mouse. The one LTR-HSC must be accompanied

by STR-HSC to supply the short term needs for blood cells
until the LTR-HSC has sufficiently proliferated to support the
development of all the blood cell types to allow for long-term
survival. The replenishing of the marrow by male donor cells

5   is validated by analysis of the marrow cells for presence of
the Y chromosome or the congenic allele Ly 5.1.

In this manner it has been shown that one LTR-HSC
can fully repopulate the marrow of a mouse. This indicates
significant self renewal of the LTR-HSC which must occur in

10   order for one cell to fully potentiate the whole marrow
system. In addition, the presence of LTR-HSC in such a
repopulated mouse can be validated by isolation of LTR-HSC
from such a repopulated mouse as well as a normal mouse (Jones
et al., supra). However, LTR-HSC may be depleted if marrow

15   cells are serially passaged through a number of lethally
irradiated mice, indicating that continuous incessant demand
for developing cells may deplete the supply of LTR-HSC. These
experiments indicate the existence of stem cell renewal and
that, under certain circumstances, stem cell renewal can be

20   overwhelmed by factors regulating development.
The process of hematopoiesis is regulated by a
group of proteins known as hematopoietic growth factors
including colony stimulating factors and interleukins. Growth
and development of hematopoietic cells is under strict

25   regulation by these factors requiring constant presence of
factor(s) for continued growth and differentiation. Different
levels and/or mixtures of factors may account for
developmental determination at points in stem cell
differentiation and maturation of progeny. The effects of

30   these factors are mediated through cell surface proteins known
as hematopoietic receptors. There are factors such as stem
cell factor (SCF), granulocyte macrophage colony stimulating
factor (GM-CSF), and macrophage colony stimulating factor
(M-CSF) which affect early, middle, and late stage in vitro

35   development respectively.
By analogy to the regulation of other hematopoietic
cells, self-renewal of stem cells is thought to be regulated

by factor(s) mediating their effect through cell surface
receptors regulating this process. To date the responsible
factor(s) is unknown.

5   The primary control mechanisms by which the
hematopoietic hierarchy is maintained are unknown. For
example, vertebrates maintain what appears to be a very finely
regulated production of all blood cell types that is generated
by a hierarchy of marrow cells that progressively give rise to
daughter cells with less proliferative and differentiation
10   potential. This hierarchy may be maintained at all levels by
varying degrees of self-replication within a "compartment,"
and also by differentiation of cells into and out of a
compartment. It would be useful to identify self-replication
factors (SRFs) in these compartments.

15   Most hematopoietic growth factors act as potent
mediators of differentiation, and as such are classic
differentiation factors (DFs). However, the most primitive
cells of the stem cell compartment are unique in that there
are no cells that can differentiate into this compartment. A
20   differentiation event occurs within the stem cell compartment
when LTR-HSC divide and generate STR-HSC which appear to have
limited clonal lifespans (see, Bartelmez et al., Exp Hematol
2:861-862 (1991)). However, the LTR-HSC appear to have an
unlimited lifespan which could be accounted for through an
25   asymmetrical division (one daughter cell is a replicate of the
mother cell, the other enters the hematopoietic pool of cells
with limited clonal lifespans). However, only a symmetrical
division of self replication would lead to clonal expansion.
Evidence that clonal expansion occurs is provided by in vivo
30   data obtained using retrovirally marked, serially transplanted
marrow, where the expansion of individual HSC clones is
clearly observed (Dick et al., Cell 42:71 (1985); Keller and
Snodgrass, J. Exp. Med. 171: 1407 (1990); Jordan and
Lemischka, Genes Dev. 4: 220 (1990)). The mechanism that
35   provides this expansion is unknown, but it may be intrinsic or
extrinsic (Wolpert, J. Cell Sci. (Suppl) 10:1-9 (1988); Ogawa
et al., Blood 81: 2844-2853 (1993)). Transforming growth

factor-beta-1 (TGF-ß1) has been identified as an extrinsic and
intrinsic (Sitnicka et al., Blood 88: 82-88 (1996)) mediator
of self-renewal of LTR-HSC.

In culture, in the presence of a known growth
factor or combination of factors, LTR-HSC do not undergo self
renewal. Culturing stem cells in the presence of cell
condition media or on feeder cells has also not been reported
to stimulate self renewal of LTR-HSC in culture (Knobel et
al., Exp. Hematol. 22: 1227-1235 (1994); van der Loo and
Ploemacher, Blood 85: 2598-2606 (1995). To confirm the
existence of such a factor, the cDNA encoding this activity
must be isolated.

There remains a need in the art for a method of
culturing hematopoietic cells which results in expansion of
the number of hematopoietic precursor cells and enhances the
yield and recovery of the precursor cells without compromising
viability. Quite surprisingly, the present invention
addresses this and other related needs.

Summary of the Invention

The present invention provides, in one aspect,
methods for increasing the number of hematopoietic precursor
cells by exposing the precursor cells to leptin, thereby
increasing the number of precursor cells relative to the
number of cells initially present. The exposure of the cells
can occur either in vitro or in vivo. The hematopoietic
precursor cells can be separated from mature hematopoietic
cells present in a blood product, such as bone marrow,
umbilical cord blood, or peripheral blood, prior to exposing
the hematopoietic precursor cells to leptin. The
hematopoietic precursor cells are stem cells or progenitor
cells. Typically the hematopoietic precursor cells which are
exposed to the leptin are lin- cells, and more preferably are
a lin-/c-kit+ fraction thereof, and include a lin-/Rh low/c-

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kit+ and lin-/Rh high/c-kit+ cellular fraction. Although
cells from a variety of mammals can be employed in these
methods, when the cells are intended for administration to a
human they will typically be human in origin. When the cells
5   are exposed to the leptin in vitro in a culture medium,
typically at least one exogenously added growth factor, such
as G-CSF, GM-CSF, IL-1, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10,
IL-11, IL-12 or TGF-β, will be included.

In another embodiment the invention provides a
10   method for maintaining the number of hematopoietic stem cells
in vitro, comprising exposing the stem cells to leptin and
culturing the cells. The number of stem cells relative to the
number of cells initially present is thereby maintained. The
hematopoietic stem cells can be separated from mature
15   hematopoietic cells present in a blood product, such as marrow
or umbilical blood, prior to exposing the stem cells to
leptin. The hematopoietic stem cells are typically lin-/c-
kit+ cells or a lin-/Rh low/c-kit+ or lin-/Rh high/c-kit+
fraction thereof.

20   In yet another aspect the invention provides a
method for sustaining primitive hematopoietic stem cells in
vitro without substantial proliferation or differentiation.
In this method the primitive hematopoietic stem cells are
exposed to leptin and then cultured, and the number of
25   primitive stem cells relative to the number of cells initially
present is sustained, e.g., for at least five to seven days.
Typically the primitive hematopoietic stem cells are lin-/Rh
low/Ho low/c-kit+ or lin-/c-kit+, and may be separated from
mature hematopoietic cells present in a blood product prior to
30   exposing the primitive hematopoietic stem cells to leptin.
The primitive hematopoietic stem cells can be subsequently
stimulated to proliferate and differentiate.

In another embodiment the invention provides a
method for inhibiting the proliferation of hematopoietic cells
35   that have been stimulated by IL-3. IL-3 stimulated
hematopoietic cells are exposed to leptin, thereby inhibiting

the proliferation of cells induced by IL-3. The hematopoietic
cells can be in fractionated or unfractionated bone marrow.

5   In yet another embodiment the invention provides a
method for increasing the number of hematopoietic cells in a
mammalian host. According to this method, the hematopoietic
precursor cells of the host are exposed to leptin, either _in_
vitro or _in vivo_, thereby increasing the number of precursor
cells relative to the number of cells initially present. When
in vitro, the hematopoietic precursor cells can be separated
10   from mature hematopoietic cells present in the blood product
such as bone marrow prior to exposing the cells to leptin.
Typically the hematopoietic precursor cells, of a lin-
fraction, such as lin-/c-kit+, are obtained from the host
prior to initiation of cytotoxic therapy.

15   A composition for sustaining or expanding the
number of hematopoietic precursor cells is also provided. The
composition comprises a nutrient medium and leptin. Typically
the composition further comprises at least one exogenously
supplied growth factor, such as G-CSF, GM-CSF, SCF, IL-1, IL-
20   3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 or TGF-β.

Brief Description of The Figures

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           Fig. 1 shows the detection of leptin receptor mRNA
expressed in bone marrow cells and hypothalamus, where 1 μg,
0.1 μg and 0.01 μg of DNA from cDNA library prepared from RNA
of hypothalamus (lanes a, d, g, respectively), bone marrow
30   (lanes b, e, h, respectively) and ALVA 31 prostrate tumor
cells (lanes c, f, i, respectively) were used as template for
PCR amplification using primers which recognize a common
sequence found in known forms of leptin receptor. The
amplified DNA was fractionated by 1 % agarose gel
35   electrophoresis, stained with ethidium bromide, excited with
UV light, and photographed. The 100 bp ladder (Pharmacia) was
used as marker DNA.

Fig. 2 shows the detection of multiple forms of
leptin receptor mRNA expressed in bone marrow, where 1 μg of
DNA from the bone marrow cDNA library was used for PCR
amplification with primers specific for each alternatively
spliced forms of leptin receptor. Lanes a, b, c, d, and e,
show Ra, Rb, Rc, Rd and Re forms of leptin cDNA, respectively,
and a 100 bp ladder was used as marker DNA.

Detailed Description Of The Specific Embodiments

The present invention provides compositions and
methods for maintaining and increasing the number of
hematopoietic precursor cells _in vitro_ and _in vivo_.
Specifically, the present invention provides methods to
sustain the growth and differentiation of hematopoietic
precursor cells _in vivo_ or _ex vivo_ by exposing the cells to
leptin or leptin-like proteins or analogs which are capable of
stimulating leptin receptor-mediated activity in these cells.
Leptin and analogs thereof can also be used in the present
invention to sustain self replication of primitive
hematopoietic stem cells _in vitro_, to inhibit IL-3 stimulated
differentiation of hematopoietic cells, and to enrich for
hematopoietic cells expressing leptin receptor in a mixed
population of hematopoietic cells. In another aspect, leptin
can be used synergistically with other exogenously added
hematopoietic growth factors to support and expand
hematopoietic stem and progenitor cells. The ability to
sustain and expand hematopoietic precursor cells, derived from
the bone marrow, peripheral blood, or umbilical cord blood of
a patient or donor, counters some of the disadvantages of
immunosuppressive or immunodestructive therapies which are
often used in the treatment of cancer and other life-
threatening diseases. For example, cultured hematopoietic
cells can be used as an important source of proliferating
cells to reconstitute a patient's blood-clotting and

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infection-fighting functions subsequent to therapy. In
addition, the ability to expand hematopoietic precursor cells
and their precursors _in vitro_ relieves dependence on bone
marrow aspiration or multiple aphereses as the only means of

5   obtaining sufficient cells for transplantation.
            For _in vitro_ use of leptin in the present
invention, hematopoietic precursor cells are typically
separated from a blood product, such as bone marrow,
peripheral blood, or umbilical cord blood of a patient or

10   donor, fetal peripheral blood and other sources. As discussed
in more detail below; such separation may be performed, for
example, by depleting the blood product of cells that are
committed to specific lineages based on their expression of
one or more antigens which are present on substantially all

15   lineage committed cells but are substantially absent from the
lineage committed precursor cells. This lin- fraction is
enriched for hematopoietic progenitor cells. The lin- cells
can be further fractionated, e.g., based on their expression
of c-kit on the cell surface into c-kit+ and c-kit- fractions,

20   as described in more detail below, and fractionated even
further based again of differential expression of cell surface
markers associated with a desired subpopulation. The
separated hematopoietic precursor cells may be stored frozen
and thawed at a later date for inoculation into a suitable

25   vessel containing a culture medium comprising a nutritive
medium and leptin, optionally supplemented with a source of
growth factors and, optionally, human or other animal plasma
or serum. Alternatively, the separated cells may be
inoculated directly into culture without first freezing. In

30   both cases the resultant cell suspension is cultured in the
presence of leptin under conditions and for a time sufficient
to increase the number of hematopoietic precursor cells
relative to the number of such cells present initially in the
blood product. The cells may then be separated by any of a

35   variety of methods, such as centrifugation or filtration, from
the medium in which they have been cultured, and may be washed
one or more times with fresh medium or buffer. Optionally,

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the cells may be re-separated into lin- and lin+ fractions
prior to resuspension to a desired concentration in a medium
or buffer suitable for infusion. The cells may then be
infused into a patient or stored frozen for infusion at a
5   later date.

Surprisingly, separated precursor cells, such as
lin- cells and lin-/c-kit+ cells, will expand in number when
cultured in the presence of medium containing leptin, enabling
clinically practicable expansion and recovery of hematopoietic
10   precursor cells. By working with separated precursor cells,
the volumes of cells and culture fluids which must be handled
are reduced to more manageable numbers. Further, a high
degree of expansion can be achieved when one starts with
separated lin- and lin-/c-kit+ cells, rather than with an
15   unseparated blood product. This is believed to be due to the
removal of cells otherwise present in the blood product, which
inhibit expansion of the precursor cells. Under the
conditions employed in the methods of this invention, cell
recovery is greatly facilitated and viability is preserved.
20   Most importantly, the yield of hematopoietic precursor cells,
capable of mediating both long-term and short-term
hematopoietic recovery in a myelosuppressed or myeloablated
host, is increased. The ability to sustain or expand
hematopoietic precursor cells _in vitro_ or _in vivo_ by the
25   compositions and methods of the present invention is expected
to have tremendously important consequences for disease
treatments which are inherently myelosuppressive or
myeloablative, such as in cancer chemotherapy.

Within the context of the present invention, the
30   lin- hematopoietic precursor (non-committed) cells include
those which do not express antigens associated with
neutrophils and activated macrophages (e.g., 7/4), B and pre-B
lymphocytes (e.g., B220), erythrocytes (YW 25.12.7),
neutrophils and macrophages (e.g., GR-1), T cells (Lyt-2), T-
35   helper cells (e.g., L3T4), nucleated red blood cells (e.g.,
Ter 119) and include primitive, totipotent stem cells as well
as progenitor cells. Lin- cells/c-kit+ cells are those lin-

cells which express c-kit antigen, as can be conveniently
determined with an antibody to c-kit. Even further
fractionation of lin- or lin-/c-kit+ cells based on amount of
rhodamine 123 fluorescence can be used to provide fractions of

5   lin-/Rh low or lin-/Rh low/c-kit+ cells containing primitive
hematopoietic cells, whereas cells of the Rh high fraction are
less primitive. The level of expression of the cell surface
antigens will vary from one cell type to another.
Consequently, a cell is operationally defined as lin-, c-kit+,

10   or Rh low, if it expresses or does not express sufficient
antigen to be detected by a given method of assay. For
example, c-kit+ cells can be identified by flow
microfluorimetry using a fluorescence-activated cell sorter
(FACS), by immunofluorescence or immunoperoxidase staining

15   using a fluorescence or light microscope, by
radioimmunoassay, or by immunoaffinity chromatography, among
numerous other methods which will be readily apparent to one
skilled in the art (see, for example, Lansdorp and Thomas (in
Bone Marrow Processing and Purging, A.P. Gee (ed.), Boca

20   Raton: CRC Press (1991) pg. 351). Hematopoietic precursor
cells can also be detected by various colony-forming assays,
such as CFU-GM and CFU-S assays (see, e.g., Till and
McCullough, supra; Bradley and Metcalf, Aust. J. Exp. Biol.
Med. Sci. 44: 287 (1966)).

25   Hematopoietic precursor cells, including lin- and
lin-/c-kit+ cells, may be obtained from any of a variety of
blood products, including bone marrow, peripheral blood,
umbilical cord blood, fetal liver, and spleen. Bone marrow is
a particularly rich source of precursor cells (1-2% of

30   marrow), but alternate sources may be preferable because of
the discomfort associated with bone marrow aspiration. Bone
marrow is typically aspirated from the iliac crest, but may be
obtained from other sites (such as the sternum or vertebral
bodies) if necessitated by prior or concurrent disease or

35   therapy. Peripheral blood contains fewer precursor cells
(typically < 1% of peripheral blood mononuclear cells), but is
generally easier to obtain than bone marrow. The number of

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precursor cells circulating in peripheral blood can be
increased by prior exposure of the donor to certain growth
factors, such as, for example, G-CSF or SCF (kit ligand (KL)),
and/or certain drugs, such as, for example, cyclophosphamide

5   or prednisone. Peripheral blood collected from patients or
donors who have been pretreated to increase the number of
circulating hematopoietic precursor cells is referred to as
having been "mobilized." According to one aspect of the
invention, leptin can be used to mobilize hematopoietic stem

10   cells from the marrow to the peripheral blood for collection.
Depending upon the volume which is desired, blood may be
obtained by venipuncture or by one or more aphereses.
Precursor cells can also be obtained from umbilical cord blood
at the time of delivery, either by simple gravity-induced

15   drainage or manual expression.

       Separation of precursor cells from more mature
cells can be accomplished by any of a variety of methods known
tp those skilled in the art, including immunoaffinity
chromatography (Basch et al., J. Immunol. Methods 56:269

20   (1983)), fluorescence-activated cell sorting, panning (Wysocki
and Sato, Proc. Natl. Acad. Sci. USA 15: 2844 (1978)),
magnetic-activated cell sorting (Miltenyi et al., Cytometry
11: 231 (1990)), and cytolysis. According to the present
invention, leptin is used to support survival of stem cells

25   during the collection and processing of whole or fractionated
marrow. Generally, separation of a heterogeneous population
of cells, such as in a bone marrow aspirate or a peripheral
blood specimen or apheresis product, into target (e.g., CD34+
or lin-) and non-target (e.g., CD34- or lin+) fractions is

30   rarely complete. For the purposes of the present invention,
separation is considered to have been accomplished if the
target fraction is comprised of at least about 20% precursor
cells, more often about 50% precursor cells, and preferably
about 70% precursor cells. In addition, it may be desirable

35   to keep the total numbers of mature hematopoietic cells, such
as platelets, granulocytes, and red cells, as low as possible
in order to prevent clumping and the release of degradative

enzymes which can adversely affect the recovery and viability
of engrafting cells, especially after freezing and thawing.
More specifically, it may be desirable that the target
fraction be comprised of less than about 5% platelets, 50%
5   granulocytes, and 10% red cells and, preferably, less than
about 1% platelets, 25% granulocytes, and 1% red cells.

Precursor cells may be positively selected or
negatively selected. By positive selection is meant the
capture of cells by some means, usually immunological, on the
10   basis of their expression of a specific characteristic or set
of characteristics (usually an antigen(s) expressed at the
cell surface). For example, CD34+ or c-kit+ cells can be
positively selected by any of the above methods (except
cytolysis, which would result in destruction of the desired
15   cells) on the basis of their expression of the c-kit antigen
utilizing an anti-c-kit antibody, such as anti-human
monoclonal antibody YB5.B8 (available from Pharmingen, Inc.),
or anti-CD34 monoclonal antibodies which are also commercially
available, e.g., monoclonal antibodies 12.8, My-10, or 8G12
20   (Becton Dickinson Co., Mountain View, CA).

Negative selection means the exclusion or depletion
of cells by some means, usually immunological, on the basis of
their lack of expression of a specific characteristic or set
of characteristics (again, usually a surface antigen). For
25   example, lin- cells can be negatively selected by any of the
above methods on the basis of their lack of expression of
lineage-defining antigens, such as CD19 (for B lymphocytes),
CD3 (for T lymphocytes), CD56 (for NK cells), CD4 (T-helper
cells), CD8 (cytotoxic T cells), CD20 (macrophages), CD11b
30   (monocytes, granulocytes, neutrophils, macrophages, NK cells,
and activated lymphocytes), CD33 (monocytes, activated T
cells, myeloid progenitors), glycophorin A (red blood cells
and erythroid precursor cells), etc., utilizing antibodies to
the above-mentioned and other lineage-defining antigens. By
35   using a cocktail or mixture of monoclonal antibodies directed
to red cell, platelet, granulocyte, lymphocyte and/or tumor
cell antigens, it is possible to leave behind a population of

cells which is highly enriched for lin- cells. Numerous
monoclonal and polyclonal antibodies suitable for this purpose
are known in the art (see Leukocyte Typing IV, Knopp et al.
(eds.), Oxford UP, 1989) and are commercially available from a
5   wide variety of sources (for example, Becton Dickinson Co.,
Mountain View, CA; Coulter Immunology, Hialeah, FL; Ortho
Diagnostics, Raritan, NJ, etc.).

Alternatively, precursor cells can be separated
from mature cells by a combination of negative and positive
10   selection techniques. A preferred combination of negative and
positive selection techniques is comprised of a first
selection for lin- cells utilizing a mixture of anti-lin+
antibodies as described above, followed by a second selection
for c-kit+/lin- cells, using an anti-c-kit antibody to a
15   determinant on the c-kit antigen. Antibodies to determinants
on the c-kit molecules are well-known in the literature (see,
e.g., Ashman et al., J. Cell. Physiol. 158: 545 (1994)) and
are available from a variety of sources, including those
mentioned above. The advantage of this or other dual
20   selection strategies is that the volume of cells which is
placed into culture is smaller, more manageable, and more
susceptible to stimulation and expansion by leptin.

During the process of leptin-stimulated expansion
according to the invention, the precursor cells are
25   periodically (e.g., every 4-7 days) separated from more mature
cells. Briefly, mature cells (which include not only
terminally differentiated blood cells, but cells of an
intermediate lineage) may inhibit the expansion and
differentiation of precursor cells via a feedback control
30   mechanism. Removal of more mature cells from a culture thus
permits expansion of the precursor cells to many times their
original numbers. Various methods may be used to periodically
separate precursor from mature cells, for example, cells can
be separated on an affinity column, incubated in a selected
35   medium, and then subsequently reseparated in order to separate
the precursor cells from the newly differentiated mature
cells. Examples of methods and devices for selecting

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precursor cells, such as CD34+, or lin-/c-kit+ cells, are
described in U.S. Patent Nos. 5,215,927, and 5,225,353, each
of which is incorporated herein by reference.

Subsequent to separation, precursor cells are
5   inoculated into a culture medium comprised of a nutritive
medium, any number of which, such as RPMI, TC 199, Ex Vivo-10,
or Iscove's DMEM, along with a source of growth factors, will
be apparent to one skilled in the art. Proliferation and
differentiation of precursor cells may be enhanced by the
10   addition of various components to the medium, including a
source of plasma or serum. Among sources of plasma or serum
are fetal bovine and human. The amount of plasma or serum
which is used will vary, but is usually between about 1 and
50% (by volume) of the medium in which the cells are grown,
15   and more often between about 1 and 25%.

According to one aspect of the present invention,
separated hematopoietic precursor cells are cultured in a
nutritive medium and leptin. The leptin is prepared as
generally described in, e.g., Friedman et al., International
20   PCT publication WO 96/05309, incorporated herein by reference.
WO 96/05309 describes the preparation of isolated and purified
leptin (alternatively described as OB polypeptide), as well as
nucleic acids encoding leptin and the amino acid sequences
thereof, including human leptin polypeptides, and peptide
25   fragments, derivatives and analogs. The leptin polypeptides
for use in the methods of the present invention can be
prepared by recombinant or synthetic means, as described in WO
96/05309. Other leptin receptor recognition molecules can
also be used in the present invention. For example, leptin
30   analogs and mimetics, including small molecule analogs, can be
identified as leptin receptor agonists or antagonists as
described in WO 96/05309. A leptin receptor has been described
in Tartaglia et al., Cell 83:1263-1271 (1995), incorporated
herein by reference. The mimetics or analogs will typically
35   have a biological activity of the leptin polypeptide,
including but not limited to specific binding to a leptin
receptor or antibody specific for a leptin molecule, or other

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recognition molecule; activation of signal transduction
pathways; and/or induction (or inhibition by antagonists) of
physiological effects mediated by the native leptin
polypeptide _in vivo_ or _in vitro_. It is understood that
5   reference herein to leptin includes molecules such as leptin
mimetics and the like which have the same or similar effect as
leptin as described herein, e.g., maintaining or expanding
hematopoietic precursor cells, sustaining self-replication of
primitive stem cells, inhibiting differentiation of
10   hematopoietic precursor cells caused by IL-3, and improving
the recovery of patients from radiation or chemotherapy.

The leptin can be used in the present invention as
a purified preparation or as a component of a composition,
including a mixture with other hematopoietic growth factors.
15   For example, among growth factors which may be employed in the
medium are interleukins (IL) 1-15, erythropoietin (US Patent
No. 4,703,008, incorporated herein by reference), stem cell
factor (SCF, also known as mast cell growth factor and c-kit
ligand), granulocyte colony stimulating factor (G-CSF),
20   granulocyte, macrophage-colony stimulating factor (GM-CSF),
macrophage-colony stimulating factor (M-CSF), transforming
growth factor beta (TGF beta), tumor necrosis factor alpha
(TNF alpha), thrombopoietin (TPO), the interferons (IFN alpha,
beta, or gamma), fibroblast growth factor (FGF),
25   platelet-derived growth factor (PDGF), insulin-like growth
factors (IGF-1 and IGF-2), megakaryocyte promoting ligand
(MPL) and SLK-2, etc. Growth factors are commercially
available, for example, from R & D Systems, Inc. (Minneapolis,
MN), Amgen, Inc. (Thousand Oaks, CA), Immunex Corp. (Seattle,
30   WA), Genetics Institute (Cambridge, MA), and Genentech, Inc.
(S. San Francisco, CA), among other sources. Particularly
preferred are combinations of growth factors, especially the
combination of IL-3, G-CSF, and SCF. In general, the
above-mentioned growth factors are purified or partially
35   purified before they are added to the culture medium.
Usually, they will be produced by recombinant DNA methods, but
they may also be purified by standard biochemical techniques

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from conditioned media. Non-naturally occurring growth
factors can also be produced by recombinant DNA methods, and
it will be evident to those skilled in the art that fusion
proteins, combining multiple growth factor activities, can be
5   readily constructed, for example, fusion proteins combining
SCF activity with that of other growth factors such as IL-1,
IL-6, G-CSF, and/or GM-CSF. The amount of each growth factor
to be used is determined empirically and will vary depending
on the purity and method of production of the factors.
10   Generally, concentrations between 0.5 and 100 ng/ml are
sufficient, more often between 0.5 and 50 ng/ml. Where more
than one growth factor is used, the optimum amount of each
factor should be determined in combination with the other
factors to be used. This is because some growth factors can
15   modulate the activity of other growth factors, necessitating
that they be used sequentially rather than simultaneously,
while in other instances, growth factors may act
synergistically. Still other growth factors may enhance
proliferation or differentiation along one pathway, while
20   suppressing another pathway of interest. For example, as
shown herein leptin suppresses the stimulation of
proliferation that is stimulated by IL-3. Thus, leptin can be
used in this situation to suppress the amplification of
certain cell types, e.g., those which are IL-3 responsive,
25   while permitting the amplification of others.

        The medium may also be prepared with or without a
source of serum or plasma. If used, the serum or plasma may
be of human or other animal origin. Particularly preferred
for cultivation of human cell is human autologous plasma or
30   human AB⁻ plasma. The amount of plasma or serum which is used
will vary, but is usually between about 1 and 50% (by volume)
of the medium in which the cells are grown, and more often
between about 1 and 25%.

        Separated precursor cells may be cultured in any
35   vessel which is capable of being sterilized, is adapted or
adaptable to gas exchange with the atmosphere, and is
constructed of a material which is non-toxic to cells. A

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variety of vessels suitable for this purpose are well-known in
the art, including stirring flasks (Corning, Inc., Corning,
NY), stirred tank reactors (Verax, Lebanon, NH), airlift
reactors, suspension cell retention reactors, cell adsorption

5   reactors, and cell entrapment reactors, petri dishes,
multiwell plates, flasks, bags and hollow fiber devices. If
agitation is desired, it can be attained by any of a variety
of means, including stirring, shaking, airlift, or end-over-
end rotation. In addition to maintaining the culture in

10   suspension by agitating the medium (as by stirring or
airlift), the culture can also be maintained in suspension by
matching the density of the culture medium to the density of
the cells or microcarrier beads.

This invention provides methods of using leptin and

15   pharmaceutical compositions comprising leptin for stimulating
hematopoiesis in mammals, including humans. These compositions
and methods are useful in promoting both long-term and short-
term hematopoietic recovery and treating side effects of in a
myelosuppressed or myeloablated host. Myelosuppression or

20   myeloablation may occur as an isolated disorder, secondary to
another disorder, or as a side effect resulting from a
specific therapeutic regimen. The leptin can be used alone or
in conjunction with other treatments, e.g., G-CSF. The leptin
is typically administered in the form of a pharmacologically

25   effective amount and a pharmaceutically acceptable carrier. A
pharmacologically effective amount is, in this case, an amount
effective to promote the proliferation of hematopoietic cells,
e.g., stem or progenitor cells, or increase the number of stem
cells in the circulating blood.

30   The form, amounts and timing of administration
generally are a matter for determination by the practitioner.
For the agents of this invention, systemic administration in a
liquid carrier by injection is preferred. In one embodiment,
the pharmaceutical composition is delivered as a unit dosage

35   form. For example, unit dosage forms for systemic
administration include unit doses of injectable solutions.
These compositions are useful in the therapeutic methods of

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this invention. Therapeutic doses generally will be in the
range of 0.1 µg/kg to 1.0 mg/kg of subject body weight per
day, preferably 1.0 µg/kg to 100 µg/kg body weight per day,
with the exact dose determined by the clinician according to
5   accepted standards, taking into account the nature and
severity of the condition to be treated, the subject's traits,
etc. Determination of dose is within the level of ordinary
skill in the art. The protein may be administered for acute
treatment, over one week or less, often over a period of one
10   to three days or may be used in chronic treatment, over
several months or years.

          For use of the present invention in bone marrow
transplantation (the transplantation of hematopoietic cells
into the bone marrow of a subject), methods are provided for
15   transplanting hematopoietic cells maintained and/or expanded
with leptin into a subject. Bone marrow transplantation is
useful for replacing hematopoietic cells in subjects whose
hematopoietic cells have been damaged or destroyed by, for
example, disease, cancer therapy or other exposure to
20   radiation. In one version of the method, bone marrow or
peripheral blood progenitor (PBPC) cells are removed from a
patient or a suitable donor prior to the patient's
chemotherapy or radiation exposure and treated with leptin,
optionally in combination with one or more other cytokines, as
25   described above. The treated marrow or PBPCs are then
returned to the patient after chemotherapy to speed the
recovery of the marrow. Prior to chemotherapy treatment,
marrow can be stimulated with stem cell factor (SCF) or G-CSF
to release early progenitor cells into peripheral circulation.
30   These progenitors can be collected and concentrated from
peripheral blood and then treated in culture with leptin,
optionally in combination with one or more other cytokines,
including but not limited to SCF, G-CSF, GM-CSF, IL-6 or
IL-11, to differentiate and proliferate into high-density
35   megakaryocyte cultures, which can then be returned to the
patient following high-dose chemotherapy.

In another aspect the present invention can be used
in methods of hematopoietic stem cell transplantation.
Procedures for obtaining hematopoietic stem cells,
readministering them and repopulating marrow are widely used.

5   To inhibit loss of "stemness" in vitro and to facilitate
survival during collection and processing, the cells are
exposed to effective concentrations of leptin during these
stages, including during cultivation. This treatment enhances
repopulation of the marrow by stem cells upon subsequent

10   readministration to the patient.

Leptin can also be used in methods of gene therapy
where hematopoietic cells are targeted for introduction of one
or more genes encoding expression product(s). Hematopoietic
stem cells are often targets of gene therapy methods, but in

15   the past it has been difficult to manipulate the stem cells in
vitro without losing the stemness of the long term
repopulating stem cells (LTR-HSC). To maintain long term
expression of a foreign gene product by gene therapy methods,
the LTR-HSC must be altered in such a way that stemness has

20   been maintained. According to this aspect of the invention,
exposure of the LTR-HSC to leptin during collection and
manipulation in vitro promotes survival and expansion of this
cell population, thereby enhancing the long term effectiveness
of the gene therapy procedure. Suitable viral vectors include

25   retroviral vectors (see Miller, Curr. Top. Microbiol. Immunol.
158: 1-24 (1992); Salmons and Gunzburg, Human Gene Therapy 4:
129-141 (1993); Miller et al., Methods in Enzymology 217: 581-
599, (1994)) and adeno-associated vectors (reviewed in Carter,
Curr. Opinion Biotech. 3: 533-539 (1992); Muzcyzka, Curr. Top.

30   Microbiol. Immunol. 158: 97-129 (1992)). Other viral vectors
that may be used within the methods include adenoviral
vectors, herpes viral vectors and Sindbis viral vectors, as
generally described in, e.g., Jolly, Cancer Gene Therapy 1:51-
64 (1994); Latchman, Molec. Biotechnol. 2:179-195 (1994); and

35   Johanning et al., Nucl. Acids Res. 23:1495-1501 (1995), each
incorporated herein by reference. The choice of vector will
rely in part on the requirement for cellular replication, the

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disease state that is being treated and the size of the gene
to be transferred.

In some instances, it may be useful to transfect
hematopoietic cells or other cells with an expression vector
5   capable of expressing leptin and introducing such cells into a
subject. Such methods are useful for increasing the amount of
leptin activity in the subject. Increasing leptin activity
would be useful in the treatment of, for example,
myelosuppression or the like. In one embodiment, the genes
10   are introduced into cells of the individual *in vivo* by means
of expression vectors. In another embodiment, the genes are
introduced into cells *ex vivo*, and transfected cells that
express and secrete leptin are administered to the subject.

The following examples are offered by way of
15   illustration, not by way of limitation.

EXAMPLE 1

20   Expression of Leptin Receptor in Bone Marrow Cells

PCR primers were initially designed according to
the mouse and human leptin receptor sequences to detect
receptor cDNA sequence in a hypothalamic cDNA library.
25   DNA from the cDNA library was first denatured by
alkaline treatment, diluted, and subjected to PCR
amplification with leptin receptor specific sequences. For
the initial experiment, a cDNA library constructed from bone
marrow tissue RNA was included as a control.
30   For isolation of RNA from tissue or cultured cells,
total RNA was extracted from mouse bone marrow tissue by the
method of Chomczynski and Sacchi, Analyt. Biochem. 162: 156-
169 (1987) and using RNAzol solution supplied by the
manufacturer (Tel-Test, Inc. Friendswood, Tx). Tissues were
35   deposited directly into appropriate volume of RNAzol solution.
Extraction of RNA followed the procedures recommended by the
manufacturer with slight modifications. Two additional

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phenol-chloroform extractions were routinely performed on the
RNA isolated by the RNAzol method to ensure the purity of RNA.

Primers for PCR were designed based on leptin
receptor sequence using the GeneWorks program
5   (IntelliGenetics). Primers were purchased from Gibco/BRL.
The PCR primer set sequence was designed to amplify sequence
from amino acid #8 through amino acid #248 at the 5' end of
the coding region of leptin receptor, and to detect all
alternatively spliced forms of leptin receptor mRNA. The pair
10   of primers had the following sequence: forward primer, 5'
TGTGGTTTTGTTACAYTGGG 3' [Seq ID No: 1]; reverse primer, 5'
TGTGAYTTCCATATGCAATCC 3' [Seq ID No: 2]. PCR was performed
with standard method recommended by the manufacturer on a Gene
Amp 2400 system (Perkin-Elmer). Taq polymerase and reagents
15   were purchased from Perkin-Elmer. PCR was generally done with
25-30 cycles of amplification. PCR product was analyzed on a
1% agarose gel.

For reverse transcription-PCR (RT-PCR), a standard
protocol was followed as described in Rao et al., FEBS Lett.
20   263: 18-22 (1990), incorporated herein by reference. Total
RNA was first reverse transcribed with either specific primer
or random primer by SuperScript II (GIBCO-BRL). The RT
reaction followed recommendations of the manufacturer. A
portion of, or diluted cDNA was used for PCR depending on the
25   abundance of the mRNA. PCR amplification was as described
above, and RT-PCR product was analyzed on an appropriate
percentage of agarose gel.

The results obtained with the hypothalamic cDNA
library and cDNA libraries constructed from bone marrow RNA
30   and from ALVA31 prostate tumor cell RNA are shown in Fig. 1.
A 720 bp band was detected from 1 μg and 0.1 μg of
hypothalamic cDNA library DNA (Fig. 1, lanes a and d).
Surprisingly, the 720 bp band was also amplified from DNA of
the bone marrow library (Fig. 1, lanes b, e, and h). The
35   intensity of the 720 bp DNA band amplified from the bone
marrow library was greater than from the hypothalamic library.
The 720 bp band could be detected from as little as 0.01 μg of

the total bone marrow library DNA. The 720 bp band was
isolated from agarose gel and partially sequenced to confirm
its identity as the leptin receptor sequence.

5   The primer set used for the initial experiments
amplified all forms of leptin receptor. Alternatively spliced
forms of leptin receptor mRNA are not revealed by this
analysis. There are at least five alternatively spliced forms
of mRNA for leptin receptor, and only one form (form b)
contains a cytoplasmic signal transducing domain. Which
10   alternatively spliced form of leptin receptor mRNA is
expressed in bone marrow tissue was determined. Specific PCR
primer sets have been used to differentially detect all five
forms of alternatively spliced mRNA (Lee et al., _Nature_ 379:
632-635 (1996)). PCR was performed with 1 ug of DNA from
15   total bone marrow cDNA library using the following primers:
for Ra, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 3],
reverse: 5' AGTCATTCAAACCATTAGTTTAGG 3' [Seq ID No: 4]; for
Rb, forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 5],
reverse: 5' TGGATAAACCCTTGCTCTTCA 3' [Seq ID No: 6]; for Rc,
20   forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 7],
reverse: 5' TGAACACAACAACATAAAGCCC 3' [Seq ID No: 8]; for Rd,
forward: 5' ACACTGTTAATTTCACACCAGAG 3' [Seq ID No: 9],
reverse: 5' AGGCTCCCTCAGGGCCAC 3' [Seq ID No: 10]; for Re,
forward: 5' TGTTATATCTGGTTATTGAATGG 3' [Seq ID No: 11],
25   reverse: 5' CATTAAATGATTTATTATCAGAATTGC 3' [Seq ID No: 12].
The results are shown in Fig. 2. Except for form d (lane d),
which can not be determined due to high background, all other
forms can be detected in the cDNA library (forms a, b, c, and
e in lanes a, b, c, and e respectively). Form b, the long
30   form is also present in the cDNA library, however, the
intensity of PCR product for form b (lane b) appears to be the
least of all forms.

          As bone marrow cells are a heterogeneous set of
cells, one goal was to identify cell types within the
35   population that express the leptin receptor mRNA by using the
RT-PCR method, based on the assumption that cells expressing
the leptin receptor will be able to respond to leptin. Cell

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lines screened included WEHI-3 cells, 32D-J558 cells, EML
cells and MPRO cells. As a result of the screening, only
WEHI-3 cells were found to express the receptor sequence.
WEH1-3 is a mouse myelomonocytic leukemia cell line. WEHI-3
5   cellular RNA was then used to determine the sensitivity of
detection by the RT-PCR method and to determine whether the
leptin receptor mRNA was the long or short form. The WEHI-3
RNA was also used to standardize and improve the sensitivity
of detection by RT-PCR method.

10   A bone marrow library was constructed with RNA
isolated from bone marrow of an animal treated with
5-Fluorouracil (5-FU) to enrich for hematopoietic stem cell
population and to determine whether 5-FU would enrich for the
cells expressing leptin receptor mRNA. 5-FU treatment ablates
15   dividing cells and therefore enriches for precursor and mature
cells of the bone marrow (Bartelmez and Stanley, J. Cell.
Physiol. 122:370-378 (1985)). Male and female F_1 hybrid mice
from the cross C57B1/6 X DBA/2 were used. Three-to-six-month
old mice came from a NIA-derived breeding colony maintained
20   under strict specific pathogen-free conditions and routinely
tested and confirmed to be free of all known mouse pathogens.
Mice of about 20 g were either treated with a single injection
of 3 μg 5-FU or not treated, and bone marrow cells were
harvested 3 days after treatment.

25   To harvest bone marrow cells, mice were sacrificed,
femurs and tibias were removed aseptically and repetitively
flushed of marrow with phosphate buffered saline (PBS), 2%
fetal bovine serum (FBS) (Biocel, Rancho Dominguez, CA). This
solution was used as collection medium and for cell
30   resuspension through lineage depletion steps described below.
The low density bone marrow cells were isolated on a gradient
by layering 5 ml aliquots of 10^7 cells/ml over 4.0 ml Nycodenz
(1.080 g/ml) (Nycomed, Oslo, Norway). Cells were centrifuged
20 min at 400 x g, collected from the interface and washed
35   twice with collection medium at 4°C.

                RNA was recovered from the marrow cells of 5-FU
treated or untreated mice, quantitated and equal amount of RNA

was subject to RT-PCR. After reverse transcription (RT),
total cDNA was then diluted to compare the sensitivity of
detection from cell population of either 5-FU treated or
untreated animals. The results showed no dramatic difference

5   between samples from animals treated with 5-FU compared to
samples from animals not treated with 5-FU. These results
indicate that expression of leptin receptor might be enriched
in the stem cell or stromal cell populations, cells which are
relatively non-proliferating and thus less affected by 5-FU

10   treatment. With identical conditions leptin receptor signal
can be detected from 200 pg of total WEHI cell RNA, but
receptor signal can be detected from 2 ng of total bone marrow
RNA.

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EXAMPLE 2
Effect of Leptin on Proliferation of Hematopoietic Cells

         This Example demonstrates that Leptin has a
20   mitogenic effect on hematopoietic cells.
         Hematopoiesis is regulated by a group of proteins
known as hematopoietic growth factors and the constant
presence of these factors is required for the proliferation
and differentiation of hematopoietic cells. The effect of

25   hematopoietic growth factors is mediated through the specific
cell surface proteins known as hematopoietic growth factor
receptors. In Example I the presence of leptin receptor
sequence was detected in the bone marrow cDNA library. To
explore the possible regulatory effect of leptin on

30   hematopoiesis, the mitogenic effect of leptin on hematopoietic
cells was assessed. In an initial experiment illustrated in
Table 1, unfractionated bone marrow cells were plated in agar
at a concentration of 5000 cells per dish under three
different growth conditions: (1) medium alone, (2) leptin

35   (added to medium), or (3) IL-3 (added to medium). Mouse IL-3
was obtained from Dr. A. Hapel, Australian National University
(the activity of IL-3 was 5 U per 1 ng). As shown in Table 1,

no colony growth occurred in medium alone, 34±5 colonies grew
in the presence of IL-3, and some clusters were noticed in
plates with bone marrow cells grown in the presence of leptin.
These results suggest that either the leptin effect on bone
marrow cells is not significant or the target cell population
is infrequent and the effect can not be seen clearly in
non-fractionated marrow.

Table 1.   Effect of leptin on the proliferation on
            unfractionated bone marrow cells

Growth Conditions	Number of Colonies per 5000 plated Cells	Number of Clusters per 5000 plated Cells
medium alone	0	0
IL-3 200 ng/ml	34±5	N.D.*
leptin 100 ng/ml	0	10-20

* N.D. = Not Determined, but <100.

        The next series of experiments used different bone
marrow fractions. Bone marrow was fractionated by depleting
the marrow of cells that are committed to specific lineages
using a cocktail of antibodies against cell surface lineage
specific antigens. This "lin- fraction" is highly enriched
for hematopoietic progenitor cells. Lineage committed cell
depletion was performed as follows:
        Three-to-six-month-old male BDF_1 mice (C57Bl/6 X
DBA/2) were sacrificed, femurs and tibias were removed
aseptically and receptively flushed of marrow with PBS with 2%
FBS. Low density cells were first isolated on a gradient by
layering 5-ml aliquots of 10^7 unfractionated cells/ml over 4.0
ml Nycodenze (1.080 g/ml) (Nycomed, Oslo, Norway). Cells were
centrifuged 20 minutes at 400xg, collected from the interface
and washed with a 10x excess of PBS with 2% FBS. Lineage
committed cells were then removed by the antibody mediated
magnetic bead depletion using the following rat anti-mouse

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monoclonal antibodies: anti-7/4 (neutrophils and activated
macrophages; obtained from S. Gordon, Oxford, UK), anti-
YW25.12.7 (blasts and nucleated erythroid cells; obtained from
I. Bertoncello, Peter MacCallum Cancer Institute, Melbourne,
5   Australia). Antibodies purchased from PharMingen (San Diego,
CA) were anti-Ter1-19 (nucleated erythroid cells), anti-B220
(B and pre-B lymphocytes), anti-Mac-1 (granulocytes,
macrophages) and anti-Gr-1 (granulocytes, monocytes). Cells
were resuspended in PBS with 2%FBS at a concentration 1×10^7
10   cells/100μl of PBS and the equal volume of antibody mix was
added (anti-YW 25.12.7 was added to the antibody mix at 10% of
the volume). After an incubation for 15 minutes at 4°C, cells
were washed by centrifugal sedimentation over a 2 ml FBS
gradient to separate cells from unbound antibodies. Cells
15   were then transferred into 50 ml centrifuge tubes (Corning)
and rosetted with magnetic beads coated with sheep anti-rat
IgG (Dynabeads M-450, Dynal, Inc., Great Neck, NJ) in the
following manner: to an equal volume of cells (350×10^6 cells/
3.5 ml maximum per tube), 10x the number of magnetic beads was
20   added dropwise to the cell suspension and mixed gently with a
pipette. The suspension was centrifuged at 20xg for 3 min,
then vigorously resuspended and transferred to a 15 ml
polypropylene tube. Then 4 ml of PBS with 10% was added to
prevent beads trapping and a tube was placed into the Dynal
25   magnet for 3 min. Cells not bound with the magnetic beads
remained in suspension and were carefully removed with a
pipette (this fraction was designated lineage negative cells,
lin-). The lin- cells were counted and resuspended in 10mM
Hoechst 33342 (Ho) (Sigma Chemical, St Louis, MO) in PBS with
30   10% FBS (adjusted to pH 7.2 with $NaHCO_3$) and incubated at 37°C
for 1 hr. After 40 min of Ho incubation, 0.1 μg/ml of
Rhodamine 123 (Rh 123) (Sigma) was added to the cell
suspension, giving 20 min total incubation with Rh 123. After
dye incubation, cells were centrifuged, chilled to 4°C, washed
35   with PBS with 2% FBS and adjusted to 3×10^6 cells per 100ml.
Cells were then incubated for 15 minutes at 4°C with directly
phycoerythrin-labeled anti c-kit antibody (2B8 clone,

PharMingen) at 1μg/ml, then washed and resuspended in PBS with
2% FBS. Finally, 2μg/ml of propidium iodide (Sigma) was added
to the cell suspension for detection of dead cells, just prior
to cell sorting.

5   At the next step of fractionation, lin- cells were
selected based on their expression of c-kit on the cell
surface into c-kit+ and c-kit- fractions. Previous data
showed that the c-kit+ fraction is highly responsive to
hematopoietic growth factors. To select for more primitive
10   hematopoietic cell fraction, lin-/c-kit+ cells were separated
based on the expression of different amount of Rhodamine 123
fluorescence into Rh low and Rh high fractions.

         The pre-fractionated cells were analyzed and sorted
on a FACStar Plus flow cytometer (Becton Dickinson, San Jose,
15   CA) equipped with dual argon lasers, and an automated cell
delivery unit (ACDU). Cells were kept chilled at 4°C with a
recirculating water bath. Monochromatic light at 351-364 nm
and 488 nm was used for Ho and Rh 123 excitation,
respectively. Forward light scatter was detected using
20   488bp10 and ND 1.0 filters. Rhodamine 123 emission was
detected using a 530bp20 filter, PE emission using a 575bp20
filter, propidium iodide emission using a 610 lp filter, and
Hoechst emission using 395bp20, 485bp15, or a 515 lp filter,
as indicated.

25   The lin-/Rh low/c-kit+ fraction contains primitive
hematopoietic cells, whereas cells in the Rh high fraction are
less primitive. The different bone marrow fractions were then
expose to leptin. In experiments illustrated in Table 2, the
single cell culture system was used to determine the direct
30   effect on the target cell population and to measure the
cloning efficiency (the proportion of dividing cells).

         In the single cell culture system, single sorted
cell were directly deposited into 96-well U-bottomed plates
(Corning) into Iscove's modified Dulbecco's medium (IMDM)
35   (Gibco-BRL, Grand Island, NY) supplemented with cytokines as
indicated and with 12.5% FBS, 12.5% horse serum (HS) (Gibco),
2×10^{-5} mol/L 2-mercaptoethanol (2ME) (Sigma), 10^{-7} mol/L

hydrocortisone (HC) (Sigma), and antibiotics (penicillin/
streptomycin) (Gibco). Growth factors were used at the
following concentrations: 50 ng/ml of SCF, 20 ng/ml of IL-6,
100 ng/ml of IL-3 in triple cytokine combination and 400 ng/ml
5   of IL-3 used as a single growth factor. In all experiments,
cells were cultured for 2-3 weeks. The use of U-bottom plates
facilitated the settling of single cells to the bottom center,
which allowed direct observation. Clones ranging from 2-64
cells could be directly enumerated. The number of cells per
10   well were directly counted using a phase contrast microscope
at 200 x magnification. Where indicated, single cell survival
was determined by trypan blue exclusion coupled with the
ability of live cells to highly refract light. Cell
differentials were performed by counting cytospin preparations
15   on slides stained by the Giemsa-Wright method.

Table 2 shows that leptin had a direct effect on
cells from both the lin-/c-kit+ and lin-/Rh high/c-kit+, bone
marrow fractions enriched for progenitor cells, where a
substantial proportion of cells (20±5% and 22±6% of cells,
20   respectively) proliferated in response to leptin. After 7
days of culture in leptin (100 ng/ml), the proliferating
clones reached the size of 100-200 cells per clone,
corresponding to 7-8 cell divisions.

In contrast, lin-/Rh low/c-kit+ bone marrow
25   fraction that contained more primitive hematopoietic cells did
not respond to leptin. These results showed that leptin has
direct mitogenic on specific bone marrow cell populations.

32.

Table 2:   Effect of leptin on the proliferation of single BM
           cells

Bone Marrow Fraction	Cloning Efficiency in medium alone	Clone Size in medium alone at day 7	Cloning Efficiency in Leptin 200 ng/ml	Clone Size in Leptin 200 ng/ml at day 7
lin-/c-kit+ *	2±2	0	20±5	80±65
lin-/Rh low /c-kit+	0±0	0	0	0
lin-/Rh high /c-kit+	1±1	0	22±6	97±57

* no cell proliferation seen in lin-/c-kit- fraction

To further determine the effect of leptin on the
proliferation of bone marrow cells, the rate of cell
proliferation was analyzed in multiple cells per well. The
above data showed that lin-/c-kit + bone marrow fraction
contains cells that are direct target cells for leptin. To
determine whether there are other target cells in this cell
fraction that produce and release growth factors in response
to leptin, lin-/c-kit+ cells were grown in the presence of
leptin at different cell concentrations for seven days and the
cell number then counted. The indicated number of lin- or
lin-/c-kit+ cells was directly deposited from the sorter into
96 U-bottomed culture plates containing medium alone or medium
with 200 ng/ml leptin. As shown in the Table 3, no
proliferation of cells was observed in medium alone. The
number of cells after seven days of culture in leptin
increased proportionally with the number of cells per well
plated at day 0. For example: 50 cells plated at day 0 grew
into 440 cells after 7 days, indicating that the cell number
expanded during the culture 8.8 fold compared to the cell
number at the beginning of the culture. There was no
significant difference in the rate of leptin induced cell
proliferation between wells containing different cell number
at the beginning of culture. Therefore, it appeared that
leptin did not have an indirect effect on cell proliferation
with lin-/c-kit+ cells.

Table 3:   Effect of leptin on the proliferation of BM cells
           in multiple cells/well cell cultures

Bone Marrow Fraction	Number of Cells/well at day 0	Number of Cells/well after 7 days in medium alone	Number of Cells/well after 7 days in leptin 200 ng/ml	Increase in Cell Number after 7 days in leptin 200 ng/ml
lin-/c-kit+	50	0	440	8.8 fold
lin-/c-kit+	250	0	2250	9.0 fold
lin-/c-kit+	500	0	4000	8.0 fold
lin-/c-kit+	1000	0	12500	12.5 fold

In contrast, in the lin- cell population leptin had
an indirect effect on cell proliferation. As shown in Table
4, the rate of the proliferative response to leptin increased
significantly with increased number of lin- cells plated in
the presence of leptin at day 0. This suggests that leptin
induces the production of other hematopoietic growth factor(s)
that stimulate proliferation on lin- cells.

Table 4:   Effect of leptin on the proliferation of BM cells
           in multiple cells/well cell cultures (data from one
           rep. experiment).

Bone Marrow Fraction	Number of Cells/well at day 0	Number of Cells/well after 7 days in medium alone	Number of Cells/well after 7 days in leptin 200 ng/ml	Increase in Cell Number after 7 days in leptin 200 ng/ml
lin-	20	0	140±96	7 fold
lin-	40	1.7±2.8	182±102	4.6 fold
lin-	80	2.3±3.2	1407±379	17.5 fold
lin-	100	4±2	2213±533	22.1 fold
lin-	200	7±6	13333±6292	66.7 fold
lin-	400	25±9	308333±18764	77.1 fold

As hematopoietic progenitor cells give rise into
functionally mature and differentiated blood cells, their
proliferation and differentiation is dependent on the presence

of specific hematopoietic growth factors. In vitro, the
number of progenitor cells can be assessed by colony formation
in agar in the presence of hematopoietic growth factors.
Thus, the effect of leptin on the generation of hematopoietic
5   progenitor cells was determined. Different bone marrow
fractions were grown in the presence of medium alone or leptin
for 7 days at the different cell concentration. After this
time, all the cells were transferred into the agar cultures
containing IL-3, IL-6, and SCF and the number of progenitor
10   cells was determined after additional 14 days of culture.
Human IL-6 (rhuIL-6) was obtained from Immunex Corp., Seattle,
WA, and rat SCF was obtained from Amgen Inc., Thousand Oaks,
CA. Table 5 shows the number of progenitor cells derived from
different bone marrow fractions in the presence of leptin. No
15   progenitors were generated from lin-/c-kit- fraction, and no
progenitors were seen in any of tested fractions after 7 days
culture in medium alone. Thus, it appears that the number of
progenitors detected in the presence of leptin was dependent
on the number of lin- and lin-/Rh high/c-kit+ plated. In both
20   of these fractions, progenitor cells were generated when 250
cells were put in the culture with leptin at day 0. In
contrast, in the lin-/c-kit+ fraction the generation of
progenitors could be measured when as few as 50 cells were
cultured in the presence of leptin. These results indicate
25   that leptin has an effect on the generation of hematopoietic
progenitor cells, either on the self replication of
progenitors cells and/or the generation of progenitor cells
from less mature pre-progenitor cells.

Table 5:   Effect of leptin on the generation of progenitor
           cells

Bone Marrow Fraction	% lin- Fraction	Number of Cells Cells at day 0	Number of Proginators at day 0	Number of Progenitors after 7 days in medium alone	Number of Progenitors after 7 days in leptin 200 ng/ml	Fold Change in Progentors Number
lin-	100	50	3*	0±0	4±1	1.3
		250	15	0±0	212±98	14.1
		500	30	0±0	325±31	10.8
		1000	60	0±0	480±149	8
			0	0	0	
lin-/c-kit-	50	50				
		250	0	0	0	
		500	0	0	0	
		1000	0	0	0	
lin-/c-kit+	50	50	5±1.5	0	90	18.0
		250	30±3	0	204	6.8
		500	60±6	0	604	10.1
		1000	120±12	0	1613	13.4
lin-/Rh low/c-kit+	5	50	36±11	1	5	0.14
		250	180±55	1	7	0.04
lin-/Rh High/c-kit+	20	50	9±4	0	0	0
		250	45±20	0	354	7.9

* number based on lin-/c-kit- + lin-/c-kit+

EXAMPLE 3

Leptin Supports Survival of a Hematopoietic Stem Cell
Fraction Without Proliferation or Differentiation

        In studies involving hematopoietic stem cells a
significant problem is manipulating stem cells *in vitro*
without the cells losing their viability and stemness. The
presence of most of the known hematopoietic growth factors
induces a proliferation of stem cells that is coupled with
cell differentiation. The following experiments demonstrate
that leptin can maintain hematopoietic stem cells without
inducing proliferation or differentiation.

In experiments summarized in Table 6, single stem
cells were cultured in the presence of medium alone or leptin
at two different concentrations for 7 days. Single lin-
/lowRh/lowHo/c-kit+ cells were directly deposited from the
sorter into 96-well U-bottomed plates containing medium alone
or medium with 100 ng/ml or 1 pg/ml leptin. The presence of
cells in the well was verified by microscopic examination.
After seven days cell viability was estimated by the exclusion
of trypan blue and/or by the ability to proliferate in
response to growth factors (50 ng/ml IL-3, 50 ng/ml SCF, and
20 ng/ml IL-6). Data represent mean values ± SD from 3-7
independent experiments, where 80-90 single cells were
analyzed. These results indicate that leptin acts as
surviving factor for a subpopulation of stem cells.

Table 6:   Effect of leptin on surviving of single lin-
            /lowRh/lowHo/c-kit+ cells

% single cells after 7 days in medium alone	% single cells after 7 days in leptin 100 ng/ml	% single cells at after 7 days in leptin 1 pg/ml
1.2±0.9	13±9	15±10

To further study the effect of leptin on
maintenance of stem cells *in vitro*, cells from different bone
marrow fractions were grown in the presence of leptin for 7
days and their proliferative potential then assayed in agar.
For HPP-CFC and GM-CFC assays, a double-layer nutrient agar
culture was used consisting of a 1 ml underlayer of 0.5% agar
plus hematopoietic growth factors: rrSCF (50ng/ml), rmIL-3
(115ng/ml), rhIL-1 (20ng/ml), rhCSF-1 (500U/ml) and 0.5 ml
overlay of 0.3% agar plus target cells in 35-mm Petri dishes
was used. Cultures were incubated at 37°C in a 5% O_2, 5% CO_2,
90% N_2 gas mixture. HPP-CFC and GM-CFC were enumerated at 14
days using a dissecting microscope at 10X magnification or an
inverted microscope at 25X magnification, respectively. HPP
colonies were identified as macroscopic colonies (>50,000
cells/colony) with diameters >1.0 mm with a dense center.

The results, shown in Table 7, confirm the results above that a subpopulation of stem cells present in lin-/Rh low/c-kit+ fraction is able to survive in leptin (added to medium) for 7 days and retain their proliferative potential. Previous experiments showed that after 7 days culture in other growth factor combinations stem cells either did not survive or differentiated (no HPP formation detected). The results of this experiments are striking because stem cells were the only cell population that survived for 7 days in leptin in lin-/Rh low/c-kit+ fraction.

Table 7

Bone Marrow Fraction	Number of Cells Plated	Number of HPP-CFC plated at day 0	Number of cells plated at day 0 in leptin 200 ng/ml	Number of HPP-CFC after 7 days in leptin 200 ng/ml	Number of Clusters after 7 days in leptin 200 ng/ml
lin-/c-kit+	250	27	250	0	204
lin-/c-kit-	250	0	250	0	0
lin-/Rh low /c-kit+	250	159	250	4	3
lin-/Rh high/c-kit+	250	43	250	0	354

EXAMPLE 4

Leptin Inhibits Cell Division In a Sub-population of IL-3 Responsive Hematopoietic Cells

The effect of leptin on the response of bone marrow cells to other hematopoietic growth factors was determined. IL-3 is an important growth factor for a wide variety of bone marrow cells that are at the different stages of hematopoietic cell differentiation. The results demonstrate that leptin has an inhibitory effect on colony formation that occurs in different bone marrow fractions in response to IL-3.

In the experiment shown in Table 8, different bone
marrow fractions were grown in agar in the presence of IL-3
and different concentrations of leptin. After 14 days of
culture, the number of colonies was estimated. The results

5 (Table 8) showed that leptin significantly inhibited the
growth of a population of IL-3 responsive hematopoietic cells
that were present in unfractionated bone marrow and in
different purified fractions. This inhibition appeared to be
dose dependent and was detected within physiological

10 concentrations of leptin.

Table 8: Effect of leptin on the colony formation in response to IL-3

Growth Conditions	Unfractionated BM Number of Colonies/5000 Cells	% of Inhibition	Post Gradient BM Number of Colonies/5000 Cells	% of Inhibition	Lineage depleted BM Number of Colonies/5000 Cells	% of Inhibition
IL-3	17±3		30±4		280±3	
IL-3 +Leptin 1 µg	15±3	12	26±4	13	220±3	21
IL-3 +Leptin 100 ng	10±3	41	16±2	47	150±4	46
IL-3 +Leptin 10 ng	14±4	18	33±3	0	240±4	14
IL-3 +Leptin 100 pg	17±4	0	31±4	0	280±2	0
IL-3 +Leptin 1 pg	15±3	0	33±2	0	290±2	0
IL-3 +Leptin 10 fg	18±6	0	28±5	0	300±5	0
IL-3 +Leptin 0.1 fg	18±1	0	27±5	0	290±5	0

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The observation that leptin inhibits a
proliferation of substantial fraction of IL-3 responsive cells
led to determining the effect of leptin on the proliferation
of individually cultured stem cells in response to IL-3.
Previous data showed that IL-3 responding cells represent
short term repopulating stem cell population (STR-HSC). As
shown in Table 9, in three independent experiments the IL-3
stimulated cell proliferation was significantly inhibited by
leptin. This inhibitory effect was direct, as observed in
single cell cultures.

Table 9:   Effect of leptin on the proliferation of
           hematopoietic stem cells in response to IL-3

Experiment #	Cloning Efficiency IL-3	Cloning Efficiency IL-3 + leptin 100 ng/ml
1	45%	29%
2	19%	10%
3	10%	4% (leptin 200 ng/ml)

In summary, the foregoing Examples clearly show
that leptin affects hematopoiesis in different ways and can
act on cells at different stages of differentiation. Starting
at the top of the bone marrow cell hierarchy, leptin acts as a
surviving factor for a subpopulation of primitive
hematopoietic stem cells. In the presence of leptin the
generation of progenitor cells occurs. Leptin also inhibits
the proliferative response to IL-3 in wide variety of bone
marrow cells, and leptin acts as direct mitogen for a
subpopulation of lin-/c-kit+ cells.

While specific examples have been provided, the
above description is illustrative and not restrictive. Many
variations of the invention will become apparent to those
skilled in the art upon review of this specification. The
scope of the invention should, therefore, be determined not
with reference to the above description, but instead should be

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determined with reference to the appended claims along with
their full scope of equivalents.

All publications and patent documents cited in this
application are incorporated by reference in their entirety

5   for all purposes to the same extent as if each individual
publication or patent document were so individually denoted.

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SEQUENCE LISTING

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        (I) TELEX:

    (ii) TITLE OF INVENTION: USE OF LEPTIN TO STIMULATE HEMATOPOIESIS

    (iii) NUMBER OF SEQUENCES: 12

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: WO
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        (C) CLASSIFICATION:

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        (A) APPLICATION NUMBER: US 08/736,793
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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTGGTTTTG TTACAYTGGG   20

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

43

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACACTGTTAA TTTCACACCA GAG   23

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTCATTCAA ACCATTAGTT TAGG   24

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGATAAACC CTTGCTCTTC A   21

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACACTGTTAA TTTCACACCA GAG   23

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGAACACAAC AACATAAAGC CC   22

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACACTGTTAA TTTCACACCA GAG   23

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGGCTCCCTC AGGGCCAC   18

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTTATATCT GGTTATTGAA TGG   23

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATTAAATGA TTTATTATCA GAATTGC   27

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WHAT IS CLAIMED IS:

        1.   A method for increasing the number of hematopoietic precursor cells, comprising:

        exposing the hematopoietic precursor cells to leptin, thereby increasing the number of precursor cells
5   relative to the number of cells initially present.

        2.   The method of claim 1, wherein the hematopoietic precursor cells are separated from mature hematopoietic cells present in a blood product prior to
10   exposing the hematopoietic precursor cells to leptin.

        3.   The method of claim 1, wherein the hematopoietic precursor cells are human.

15   4.   The method of claim 1, wherein the hematopoietic precursor cells are hematopoietic stem cells.

        5.   The method of claim 1, wherein the hematopoietic precursor cells are hematopoietic progenitor
20   cells.

        6.   The method of claim 1, wherein the hematopoietic precursor cells are lin- cells.

25   7.   The method of claim 6, wherein the hematopoietic precursor cells are lin-/c-kit+ cells.

        8.   The method of claim 7, wherein the hematopoietic precursor cells are lin-/Rh low/c-kit+ cells.
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        9.   The method of claim 7, wherein the hematopoietic precursor cells are lin-/Rh high/c-kit+ cells.

        10.   The method of claim 2 wherein the blood
35   product is bone marrow, umbilical cord blood, or peripheral blood.

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11.   The method of claim 1, wherein the
hematopoietic precursor cells are exposed to the leptin *in
vitro*.

5   12.   The method of claim 1, wherein the
hematopoietic precursor cells are exposed to the leptin *in
vivo*.

13.   The method of claim 11, wherein the
10   hematopoietic precursor cells are exposed to the leptin *in
vitro* in a culture medium including at least one exogenously
added growth factor.

14.   The method of claim 13, wherein the growth
15   factor is G-CSF, GM-CSF, SCF, IL-1, IL-3, IL-4, IL-6, IL-7,
IL-9, IL-10, IL-11, IL-12 or TGF-β.

15.   A method for maintaining the number of
hematopoietic stem cells *in vitro*, comprising:
20   exposing the hematopoietic stem cells to
leptin and culturing the cells, thereby maintaining the number
of stem cells relative to the number of cells initially
present.

25   16.   The method of claim 15, wherein the
hematopoietic stem cells are separated from mature
hematopoietic cells present in a blood product prior to
exposing the hematopoietic stem cells to leptin.

30   17.   The method of claim 16, wherein the
hematopoietic stem cells are human.

18.   The method of claim 17, wherein the
hematopoietic stem cells are lin-/c-kit+ cells.
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19.   The method of claim 16 wherein the blood
product is bone marrow or umbilical cord blood.

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20. A method for sustaining primitive
hematopoietic stem cells *in vitro* without proliferation or
differentiation, comprising:
          exposing the primitive hematopoietic stem
cells to leptin and culturing the cells, thereby sustaining
the number of primitive stem cells relative to the number of
cells initially present.

21. The method of claim 20, wherein the primitive
hematopoietic stem cells are lin-/Rh low/Ho low/c-kit+ or lin-
/c-kit+.

22. The method of claim 20, wherein the primitive
hematopoietic stem cells are separated from mature
hematopoietic cells present in a blood product prior to
exposing the primitive hematopoietic stem cells to leptin.

23. The method of claim 20, wherein the primitive
hematopoietic stem cells are sustained for at least five days.

24. The method of claim 20, wherein the primitive
hematopoietic stem cells are subsequently stimulated to
proliferate and differentiate.

25. A method for inhibiting proliferation of
hematopoietic cells stimulated by IL-3, comprising:
          exposing the IL-3 stimulated hematopoietic
cells to leptin, thereby inhibiting the proliferation of cells
induced by IL-3.

26. The method of claim 25, wherein the
hematopoietic cells are in unfractionated bone marrow.

27. The method of claim 25, wherein the
hematopoietic cells are precursors separated from mature
hematopoietic cells present in a blood product prior to
exposing the hematopoietic precursor cells to leptin.

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28. A method for increasing the number of
hematopoietic cells in a mammalian host in need thereof,
comprising:

exposing the hematopoietic precursor cells of
the host to leptin, thereby increasing the number of precursor
cells relative to the number of cells initially present.

29. The method of claim 28, wherein the
hematopoietic precursor cells are exposed to leptin *in vitro*.

30. The method of claim 29, wherein the
hematopoietic precursor cells are separated from mature
hematopoietic cells present in a blood product prior to
exposing the hematopoietic precursor cells to leptin.

31. The method of claim 30, wherein the
hematopoietic precursor cells are obtained from the host prior
to initiation of cytotoxic therapy.

32. The method of claim 30, wherein the
hematopoietic precursor cells are lin- cells.

33. The method of claim 32, wherein the
hematopoietic precursor cells are lin-/c-kit+ cells.

34. The method of claim 30, wherein the blood
product is bone marrow.

35. The method of claim 28, wherein leptin is
administered to the mammalian host and the hematopoietic cells
are exposed *in vivo*.

36. The method of claim 35, wherein the leptin is
administered intravenously or intramuscularly.

37. A composition for sustaining or expanding the
number of hematopoietic precursor cells which comprises a
nutrient medium and leptin.

5   38. The composition of claim 37, further
comprising at least one exogenously supplied growth factor.

39. The composition of claim 38, wherein the
growth factor is granulocyte colony stimulating factor or stem
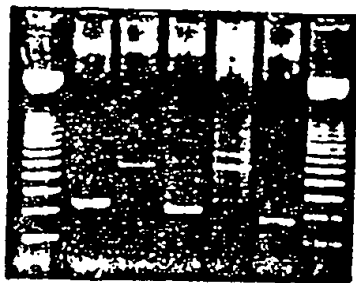10   cell factor.

Fig. 1

Fig. 2

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/00, 38/18, 38/00, CO7K 14/00

US CL : 424/198.1, 85.1; 514/12; 530/399

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1, 85.1; 514/12; 530/399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, REGISTRY, CA, BIOSIS, BIOTECHDSMEDLINE, EMBASE, LIFESCI, WPIDS, CONFSCI, DISSABS, SCISEARCH

search terms: leptin receptor, leptin, hematopoiesis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BENNETT et al. A role for leptin and its cognate receptor in hematopoiesis. Current Biology. 01 September, 1996. Vol 6. No. 9 pages 1170-1180, especially Abstract.	1-5, 10-12 -------------- 6-9, 13-39
Y	PARK et al. Primitive human hematopoietic precursors express Bcl-x but not Bcl-2. Blood. 01 August 1995. Vol 86. No. 3. pages 868-876, see entire article.	6-9, 13-39

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 DECEMBER 1997	0 3 FEB 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized Officer
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